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动物分类学报

APHELENCHOIDES DALIANENSIS SP. NOV. (NEMATODA, APHELENCHOIDIDAE) FROM PINUS THUNBERGII FROM CHINA

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Abstract A new species of nematode, *Aphelenchoides dalianensis* sp. nov. was extracted grom a wilt black pine (Pinus thunbergii) from Laotieshan (38.8°N, 121.2°E), Dalian City, China. *A. dalianensis* was characterized by its length (females: 571.5-658.0 μm; males: 436.8-520.0 μm); slender stylets (females: 10.0-12.7 μm; males: 9.2-11.8 μm) with basal knobs; 4 lateral lines. Female vulva post median, at 60%-75% of the body length; female tail with a special mucronate structure which had a divarication, forming an antennae-like terminus; male tail strongly hooked ventrally to from the characteristic 'walking-stick' form, tapering to a single terminus; small spicule (10.0-12.9 μm); 3 pairs of caudal papillae and bursa absent. *A. macronudeatus* resembles the new species, especially in male spicule, but differs from female tail terminus and the shape of male after heat-killed. The PCR-ITS-RFLP pattern and its rDNA sequence also provided further evidence that this isolate is 1 new species. *Aphelenchoides dalianensis* sp. nov. was named by the name of city, Dalian, where it was found.

Key words Nematoda, Aphelenchoididae, *Aphelenchoides*, new species.

Up to now, there are more than 180 species under genus *Aphelenchoides* Fischer (1894) described (Steiner, 1932; Christie, 1942; Sanwal, 1961; Nickle & hooper, 1991; Hunt, 1993; Liu, 2004; Xie, 2005). This genus is the largest one under Aphelenchida, and has broad hosts as well as its distribution (Hunt, 1993).

Due to the devastating pathogenicity of *A. besseyi*, *A. ritzemabosi*, *A. fragariae* to rice, chrysanthemum and strawberry, nematodes in genus *Aphdenchoides* have been studied worldwide. To our knowledge, only a few species of *Aphelenchoides* inhabit in pines and most of them unrelated to parasitism.

In 2008, we noticed some dead black pines (*Pinus thunbergii*) in Lao Tieshan, Dalian City, China, and a strange species, without bursa, was found instead of pine wood nematode. It's proved to be 1 new species of *Aphelenchoides* and here we gave a description to it.

1 Materials and Methods

Nematode. Isolated by Baermann funnel method from the cut slices of the pine wood. Then about 100 nematodes with similar shape were picked into the mycelium of *Botritis cinerea* on the PDA medium for further culture at 25℃. The isolate was further purified by breeding one pregnant female in a Petri dish with fresh mycelium of *Botritis cinerea*. Then purified specimens were killed by incubating at 65℃ for 90 second, fixed in TAF for further investigation. All the specimens used for morphological measurement were mounted in permanent slides. ITS-rDNA-RFLP Analysis. The method of DNA extraction refers to the published protocol with some modification (Subbotin *et al.*, 2000).

At first, 2 or 3 nematodes were picked into 15 μL ddH_2O and each was cut into 3 segments using No. 3 scalpel. Then all the nematode segments with 12 μL ddH_2O were transferred into 1.5 mL PCR tubes. 1.5 μL 10 × PCR reaction buffer and 1.5 μL proteinase K (1 000 μg/mL) were added into each PCR tube to complete total volume of 15 μL. The tubes were frozen at –70℃ for at least 30 min. At last, the tubes were incubated at 65℃ for 60 min and at 95℃ for 15 min.

The PCR reaction system (50 μL) contained 2 mmol/L of $MgCl_2$, 0.1 mmol/L dNTPs, 2 units of Taq polymerase (Hoyer *et al.*, 1998) and 0.6 μmol/L of each primer, the forward primer was 5′-CGTAAG-AAGGTAGCTGTAG-3′ (Ferris *et al.*, 1993) and the reverse primer was 5′-TTTCACTCGCCGTTA-AGG-3′ (Vrain, 1993). The PCR reaction was carried out as the following procedure: 95℃ for 5 min; 40 cycles of 95℃ for 30 sec, 47℃ for 30 sec, 72℃ for 2 min; and then 72℃ for 5 min. 3 μL of the PCR amplified product was used for electrophoresis to check if the concentration of the amplification product was enough for the enzyme-cutting.

Suitable tube of amplification product was chosen and digested by restriction endonuclease RsaⅠ, HaeⅢ, MspⅠ, HinfⅠ and AluⅠ with each enzyme cutting 9 μL PCR product. Enzyme-cutting fragments were resolved on 2% agrose gel and stained with 1% ethidium bromide. The details of constructing the ITS-RFLP profile referred to the published protocol (Hoyer *et al.*, 1998; Braasch *et al.*, 1999).

rDNA sequence. About 80 μL PCR product and 60 μL primers were sent to Genscript Corporation (Nanjing) for sequencing. Then the sequence was compared in GenBank to check whether this kind of nematode had been published or not.

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2 Taxonomy

Female. Body slender, cylindrical, slightly ventrally curved when heat-killed (Fig. 1). Lip region high with 6 lips of apparently equal size, offset with body by distinct constriction, labial annules present (Figs. 3, 7, 11). Stylet was 9.2-12.7 μm long, slender, with weak basal thickening. Stylet cone was less than half of total stylet length. Median bulb oval, occupying more than 3/4 of body diameter, with distinct valve situated centrally. Excretory pore very faint, behind median bulb. Dorsal esophageal gland was strong, about 4 body diameter long, overlapping intestines (Figs. 3, 7). Reproductive system was single and prodelphic. Gonad outstretched, developing oocytes in single or two files. Postmedian vulval had no flap (Fig. 13). Postuterine sac was about 1/4 of the vulva-anus distance, containing spermatozoa (Figs. 2, 8). Tail slightly curved ventrally (Figs. 6, 10), with a special structure on tail terminus. It had one mucronate structure at the end of tail, and there was a divarication on this structure, just like a snail's antennae (Figs. 6, 10, 14). 4 lateral lines (3 ridges) in the midbody (Figs. 4, 12).

Male. Body showed J-shaped after heat-killed (Fig. 1). Anterior region was similar to the females. Lateral region with 4 incisures in the midbody. Spicule was small, 10.0-12.9 μm long, the rostrum and apex were well developed. Tail strongly hooked ventrally to form as a 'walking-stick', tapering to a single terminus (Figs. 5, 9). Bursa absent. There are 3 pairs of caudal papillae, 1 pair adanal, 1 pair subterminal and the other in between.

Aphelenchoides dalianensis **sp. nov.** (Figs. 1-15)

Aphelenchida, Aphelenchina, Aphelenchoididae, Aphelenchoidinae, *Aphelenchoides* (Hunt, 1993).

A. dalianensis is characterized by its relatively short length, female's postuterine sac, especially antennae-like tail terminus, male's tail and spicule form, and 4 lateral lines. The new species has outstanding characteristics which distinguish it from other species of *Aphelenchoides*.

Etymology. The new species was named by the name of city, Dalian, where it was found.

Relationships. Nematodes in *Aphelenchoides* are separated into 4 groups by the shape of their tails (Shahina, 1996). Nematodes of group 1 have simple tails without any outgrowth or mucronate structure. Group 2 has tail with one or sometimes two mucronate structures on tail terminus. Group 3 has tail with spine or star shape terminus while group 4 has tail outgrowth other than spine or star. The new species is classified to group 2.

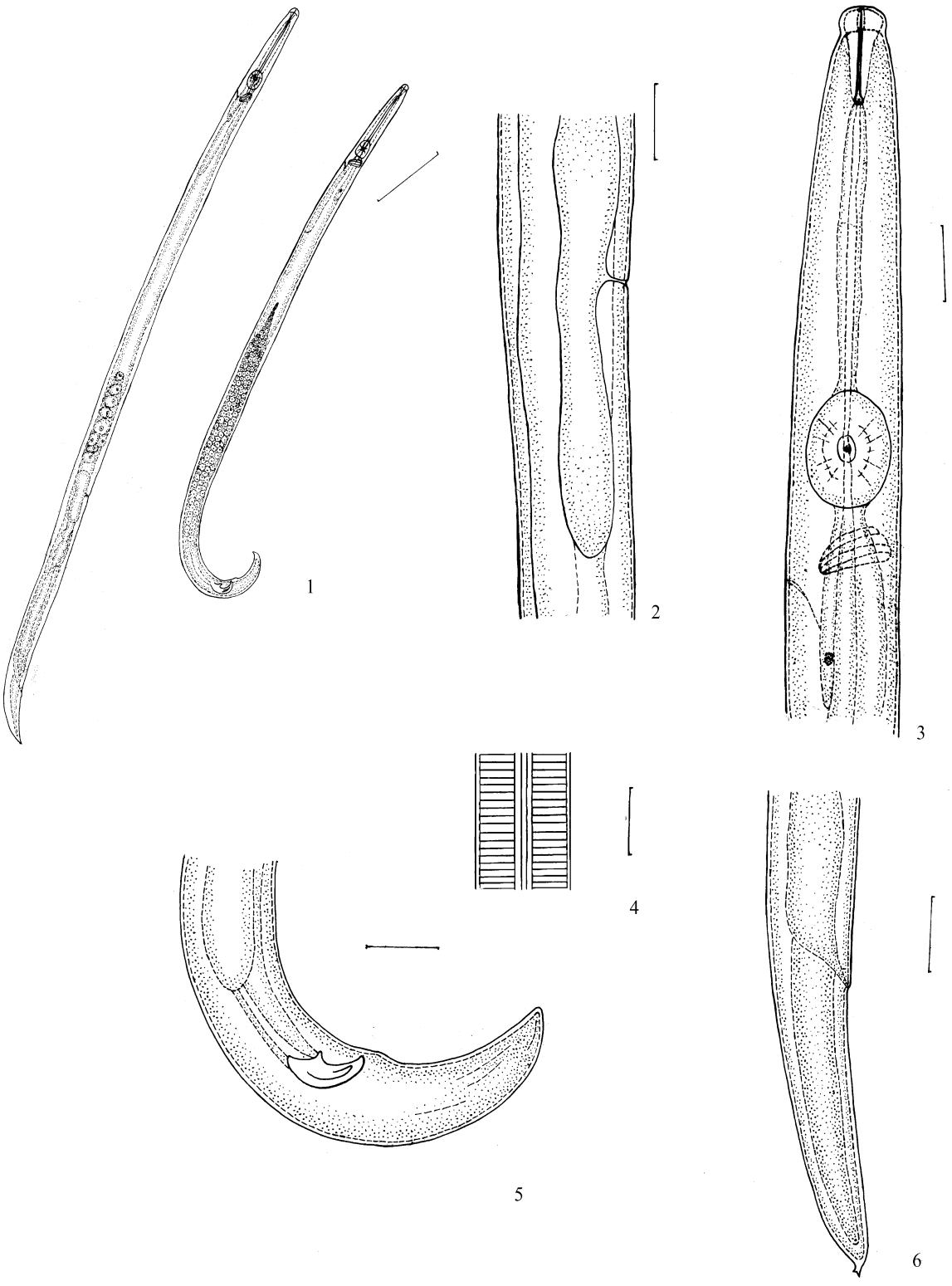
Table 1. Morphometrics of *A. dalianensis*. **Measurements in μm and in form: mean±standard deviation (range).**

	Male		Female	
	Holotype	Paratypes	Allotype	Paratypes
n	—	15	—	15
L	479.8	489.2±22.3 (436.8-520.0)	595.8	619.9±24.7 (571.5-658.0)
a	33.8	29.6±2.3 (26.3-33.8)	33.4	35.4±1.4 (33.1-37.8)
b	5.3	5.4±0.3 (5.0-6.3)	6.2	6.24±0.34 (5.67-6.70)
c	15.4	17.4±1.2 (14.7-19.6)	17.5	18.4±1.2 (17.0-20.7)
c′	2.8	2.5±0.2 (2.3-3.0)	2.9	3.2±0.3 (2.8-3.7)
V	—	—	71.4	70.9±0.6 (69.5-71.8)
Tail length	31.2	28.2±1.6 (25.1-31.2)	34.1	33.81±2.45 (29.3-39.2)
St	10.0	11.1±0.9 (10.0-12.7)	11	11±0.7 (9.2-11.8)
Sp	10.8	11.4±0.9 (10.0-12.9)	—	—
V-a	—	—	136.0	146.9±16.1 (132.2-157.8)

St: Stylet; Sp: Spicule; V-a: Vulva-anus distance. * Spicule measured along the middle line.

The new species appears to be closest to *A. macronuleatus* (Baranovskaya, 1963), which has almost the same spicule with the new species. They look the same and belong to group 2, and are both isolated from pine wood. Actually, *A. macronuleatus* has relatively longer length (630-740 μm vs 436.8-658.0 μm), longer tail (40 μm vs 25.1-39.2 μm), one simple mucronate on its tail terminus, and male tail hooks slightly to form an 'L' form after heat-killed, while the male of our new species looks like a 'J'.

A. composticola (Franklin, 1957) and *A. biaudatus* (Lmamura, 1931) are sometimes found in pine wood or soil around pine root. *A. composticola* belongs to group 4, has only 3 lateral lines and a longer spicule (21 μm vs 10.0-12.9 μm). *A. biaudatus* is sometimes found in soil around pine root. It has 2 outgrowths on tail terminus, but not like antennae, not a divarication on one outgrowth. Moreover, it has 2 lateral lines and its length



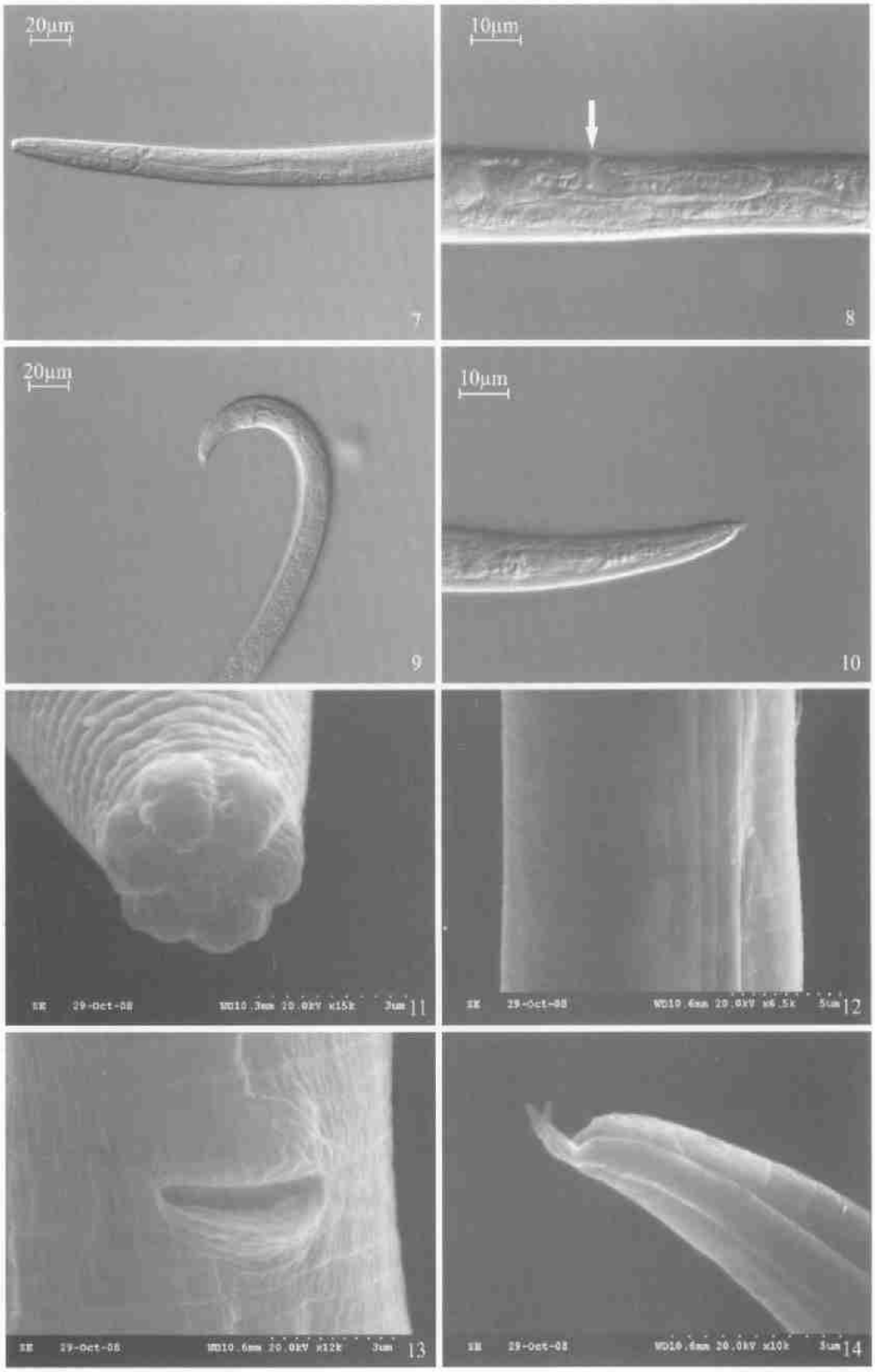
Figs 1-6. *Aphelenchoides dalianensis* sp. nov. 1. Whole bodies of female and male. 2. Vulval region. 3. Anterior part. 4. Lateral lines. 5. Male tail. 6. Female tail. Scale bars: 1= 50 μm; 2-6= 10 μm.

is relative shorter (380-470 μm vs 436.8-658.0 μm) but tail is longer (50 μm vs 25.1-39.2 μm).

A. hainanensis (Rahm, 1938; Goodey, 1951), *A. kungradensis* (Karimova, 1957) and *A. lilium* (Yokoo, 1964) also have tails which split into 2 parts. The point is that these species have divarication at the end of tail, so they are considered to have 2 mucronate structures on tail terminus. *A. hainanensis* has a longer length (900-1 300 μm vs 436.8-658.0 μm) and a larger spicule (17.5-19.0 μm vs 10.0-12.9 μm). *A. kungradensis* and *A. lilium*

belong to group 4, and they are both larger nematodes.

More details on the morphometrics of the published species similar to the new species are given in the paper. (Unit: μm, Table 2)



Figs 7-14. *Aphelenchoides dalianensis* sp. nov. 7-10. Light microscope. 7. Anterior part. 8. Female vulva. 9. Male tail. 10. Female tail. 11-14. Scanning electronic microscope. 11. Head. 12. Lateral field. 13. Female vulva. 14. Female tail.

Molecular analysis. The rDNA-PCR-RFLP pattern of the new species differed from those of *A. macronudeatus*, *A. composticola*, *A. bicaudatus* and so on (Fig. 15, Table 3). The rDNA sequence of the new species was compared with other sequences which had been published in GenBank, and this sequence was proved to be unique. The rDNA sequence of the new species was attached at the end of this paper. (Attachment 1)

Table 2. Key morphometrics of Aphelenchoides **spp. Morphologically similar to the new species. [Data (unit: μm) from published papers: Shahina, 1996]**

Species	Group	L	a	Tail	Stylet	Spicule	L. L
*A. dalianensis**	2	436.8-658.0	26.3-33.8	25.1-31.2	10.0-12.7	10.0-12.9	4
A. macronudeatus	2	630-740	34-36.3	40	11-12	—	4
A. composticola	4	440-610	30-42	35.7	11	21	3
A. bicaudatus	2	380-470	31.3-31.7	50	10-12	14	2
A. hainanensis	2	900-1300	42.4-46.1	—	10.5-11.3	17.5-19.0	—
A. kungradensis	4	650	37.6	36.7	10.7	—	4
A. lilium	4	640-750	25.8-34.5	—	12.5	17.5	4

L. L: Lateral lines.

Attachment 1. rDNA sequence of the new species.

AATAAATAATTCAATTATGCTTTGCGATTGGGGCGCATTGCATGATTTCGTAGTAGACAGTGCGGATATTCGACGGAGT-
TCGTGAAGTACTGGCTATTGCGAATGCTGGCTCTTCGCTGCGGCGTTGGGTTTCAGTCGAAATTCTAACGGCTTTTCT-
GGGCCTCTAAGTTGGATTGAGCAGTTGTGTTCCACGTCCGTGGCTGCAAAGACATCTGACGGTAGCGTTTTAGTCGCT-
TAGGTGACAGGATGCTAGAGTTGATGACCCGGTCGGGCACCCAGAACCATCATACACATTTATACAAATACATTCAATG-
AATAGAAAGTCAAGTTATGTCGGTGGATCACTTGGCTCGTGGGTCGATGAAGAACGCAGTGAATTGCGTTAATAAGCAG-
GAATTACAGATATTACGAGTGCCTTGTTTTTGATTGCATATTGCGTCGTTGGGTTTTGCCCTTCGACATACACGACTCA-
GGGTGTGTTAACGAGACACGGAAACCCGCCAAATATTGTGTTTGGTTTCCACAATAGCGATCGTCGGCTCTTCCCCAG
GGAATGTAGCTGATGTTTGTTACAGGCGGTGGTAGTGCGCCTAGATATACGAGTGTAGCGCTGTACGATTAAACGTCT-
CTCGACGAGTAAGAAACTTGTTTTCAACGAGAAACGGTCTCAGTTAATTTTGTCGAACAACTTGATGCGTAAGGAAGAT-
TTGTTCGAAACTGATGCGTTTCAATTCGGGAACAGGTTTGAGAGACGGCAGTGCAAAAGAAAACACCACTCCACACGAA-
AAATTATGTTGGTCGAGTACCCCCTGAGTTGCGTATGAGTACCTGCTGGAACTCAAGCATATCAGTAAGCAGAGGAGAA-
GAAACTAACACGGATTCTCTTATTAACCGCGCGTGTA

Table 3. Restriction fragments of the amplified PCR product of new species.

PCR production	Restriction fragments				
	Rsa I	Hae III	Msp I	Hinf I	Alu I
1 000	600	600	700	750	650
	250	325	300	250	350
	150	75			

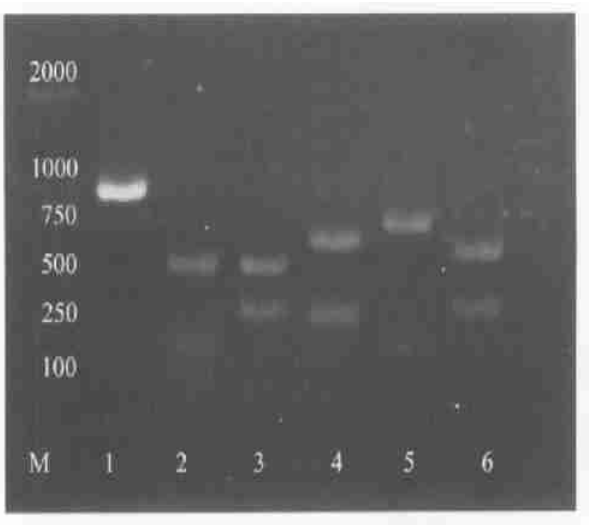


Fig. 15. The ITS-RFLP pattern of *Aphelenchoides dalianensis* sp. nov. Lane M: marker. Lane1: PCR product of ITS regions of *A. dalianensis*. Lane 2, 3, 4, 5, 6: the enzyme-cutting fragments of PCR product digested with RsaI, HaeIII, MspI, HinfI, AluI respectively.

Type host and locality. This kind of nematode was isolated from several dead black pines in Lao Tieshan, Dalian, China. Its distribution in China and abroad is not clear.

Type material. Now all the permanent slides are deposited in the Nematology Laboratory of Nanjing Agricultural University, Nanjing, China.

3 Discussion

The new species of nematode was found in Lao Tieshan, Dalian, China. Because Dalian is a seaport, we can not be sure whether this species is autochthonous or exotic. In posterior experiments, the new species could parasitize in Masson pines (*Pinus massoniana*) as well as in black pines. As a result, it is necessary to carry out Pest Risk Analysis, to investigate its distribution, host range and risk.

During molecular analysis, though the DNA quantity of only one nematode was enough for the PCR-ITS-RFLP analysis, using 2-3 nematodes gave clearer patterns.

This kind of nematode could be distinguished

quickly by its male tail, spicule form, and especially, female tail, which has an antennae-like terminus, only if it's been magnified more than 400 times.

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大连滑刃线虫新种 (线虫门, 滑刃科) 记述

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摘 要 新种大连滑刃线虫 *Aphelenchoides dalianensis* sp. nov. 采自中国辽宁省大连市老铁山的枯死黑松。新种的鉴别特征为: 体较短 (雌虫: 571.5~658.0 μm; 雄虫: 436.8~520.0 μm), 口针纤细 (雌虫: 10.0~12.7 μm; 雄虫: 9.2~11.8 μm) 具有基部球, 侧线 4 条。雌虫阴门位于虫体 60%~75% 处, 尾型特殊, 具蜗牛触角状分叉的尾尖突; 雄虫尾部向腹面弯曲成拐杖形, 有 1 简单尾尖突, 交合刺小 (10.0~12.9 μm), 乳突 3 对, 无交合伞。新种的近似种是大核滑刃线虫 *A. macronucleatus*, 主要区别在于大核滑刃线虫的雌虫仅具一简单尾尖突, 雄虫加热杀死后呈“L”形, 而非新种的“J”形。应用限制性酶切图谱 (PCR-ITS-RFLP) 的方法以及 DNA 测序为新种提供了分子生物学的证据。

词源: 新种是以标本采集地所在的城市大连命名。

关键词 线虫门, 滑刃科, 滑刃属, 新种.

中图分类号 Q959.171